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ORIGINAL RESEARCH

LXY6090 – a novel manassantin A derivative – limits breast cancer growth through hypoxia-inducible factor-I inhibition

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Abstract: Hypoxia-inducible factor-1 (HIF-1) represents a novel antitumor target owing to its involvement in vital processes considered hallmarks of cancer phenotypes. Manassantin A (MA) derived from *Saururus cernuus* has been reported as a selective HIF-1 inhibitor. Herein, the structure of MA was optimized to achieve new derivatives with simple chemical properties while retaining its activity. LXY6090 was designed to replace the central tetrahydrofuran moiety of MA with a cyclopentane ring and was identified as a potent HIF-1 inhibitor with an IC₅₀ value of 4.11 nM. It not only inhibited the activity of HIF-1 in breast cancer cells but also downregulated the protein level of HIF-1 α , which depended on von Hippel–Lindau for proteasome degradation. The related biological evaluation showed that the activity of HIF-1 target genes, *VEGF* and *IGF-2*, was decreased by LXY6090 in breast cancer cell lines. LXY6090 presented potent antitumor activity in vitro. Furthermore, LXY6090 showed in vivo anticancer efficacy by decreasing the HIF-1 α expression in nude mice bearing MX-1 tumor xenografts. In conclusion, our data provide a basis for the future development of the novel compound LXY6090 as a potential therapeutic agent for breast cancer.

Keywords: hypoxia-inducible factor-1, manassantin A derivative, antitumor, breast cancer, LXY6090

Introduction

Most solid human tumors contain areas of necrosis in which tumor cells die because of lack of oxygen.¹ A major mechanism mediating adaptive responses to reduced oxygen (hypoxia) is the regulation of the transcription factor hypoxia-inducible factor-1 (HIF-1).^{2,3} For instance, in tumor cells under hypoxia, upregulated HIF-1 can activate a series of genes that ultimately promote the malignant growth of a tumor by regulating cancer cell survival, metabolism, angiogenesis, invasion, and resistance to radiation and chemotherapy.^{4,5} Clinically, overexpression of HIF-1 has been shown in many human solid tumors and is associated with a high rate of patient mortality.

HIF-1 is a heterodimeric protein composed of an oxygen-regulated HIF-1 α subunit and a constitutively expressed HIF-1 β subunit.^{6,7} Under normoxic conditions, the prolyl-4-hydroxylases employing oxygen, ferrous iron, and oxoglutarate as substrates hydrolyze HIF-1 α at proline residues (Pro402 and Pro564).⁸ The hydroxylated HIF-1 α is recognized by a von Hippel–Lindau (VHL) tumor suppressor protein, which recruits an E3 ubiquitin-protein ligase complex; thus, the HIF-1 α subunit is kept low by abundant ubiquitination and subsequent proteasome degradation.⁹ However, during hypoxic conditions, the two proline residues cannot be hydroxylated, resulting in HIF-1 α accumulation.^{10,11} The stabilized HIF-1 α protein translocates to the nucleus and couples

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OncoTargets and Therapy downloaded from https://www.dovepress.com/ For personal use only. with HIF-1 β . Once the HIF-1 $\alpha/1\beta$ heterodimer is formed, it binds to a hypoxia-response element (HRE) in hypoxia target genes such as vascular endothelial growth factor (VEGF) and insulin-like growth factor 2 (IGF-2), which stimulate angiogenesis and cell proliferation.^{12,13}

Given the potential of HIF-1 as a cancer drug target, numerous studies have been conducted to develop HIF-1 α inhibitors.14-16 Among the small molecular inhibitors, manassantin A (MA) isolated from Saururus cernuus was recently identified as a selective and potent HIF-1 α inhibitor through a cell-based luciferase reporter assay,17-20 but its in vivo antitumor activity has not yet been evaluated. Structurally, MA contains a central tetrahydrofuran core surrounded by multiple chiral centers.^{18,21} This stereoisomeric complexity limits the feasibility of total MA synthesis and its applications as a chemotherapy agent. However, by using MA as the lead compound for development of novel HIF-1 α inhibitors, we have synthesized a series of MA derivatives based on the knowledge of their structure-activity relationship.^{22,23} For instance, LXY6099 and LXY6006 have been developed with in vivo antitumor activity. LXY6090 and LXY6099 have amide bonds, which are much easier to synthesize than LXY6006. We found that LXY6099 and LXY6006 exhibited some toxicity in in vivo mouse studies. However, we still expected to find a new MA derivative with high HIF-1 inhibition effect and low toxicity. With this background, we designed a novel MA derivative, LXY6090, to replace the tetrahydrofuran moiety of MA with a cyclopentane ring. The biological activity of LXY6090 as an active inhibitor of HIF-1 was evaluated in cell-based assays. We further explored the in vitro and in vivo antitumor activities of LXY6090 in this study.

Materials and methods Cell culture and hypoxia treatment

The current study with all the human cell lines was approved by the Research Ethics Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. Estrogen receptor-positive human breast cancer T47D and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). T47D and MCF-7 cells were cultured in Roswell Park Memorial Institute 1640 and Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) media, respectively. Estrogen receptor-negative MX-1 cells were kindly provided by Dr Yongkui Jing (Mount Sinai School of Medicine, New York, NY, USA) and maintained in Dulbecco's Modified Eagle's Medium. All these cells were cultured in a medium containing 10% fetal bovine serum (Thermo Fisher Scientific) in a humidified 5% CO₂ incubator at 37°C. Before conducting the experiments, all cells were cultured to 80%–90% confluence in six-well plates. For hypoxia induction, fully confluent plates were incubated in a hypoxic humidified incubator with a 94:5:1 mixture of N₂:CO₂:O₂. LXY6090 with high purity was stocked in dimethyl sulfoxide at a concentration of 10^{-2} M.

Luciferase reporter assay

T47D cells were plated onto 96-well plates in culture medium without antibiotics supplemented with 100 μ L serum. For each well, 0.01 μ g pRL-CMV (internal control, renilla luciferase; Promega Corporation, Fitchburg, WI, USA) and 0.2 μ g pGL2-TK-HRE (firefly luciferase) plasmids were used as previously described.^{17,24} Transient transfection and luciferase reporter gene assay (Dual-Luciferase[®] Reporter Assay System Kit; Promega Corporation) were carried out following the manufacturer's instructions. HIF-1 activities were defined as the ratio of renilla luciferase/firefly luciferase. Concentration of compounds that inhibited HIF-1 activities by 50% (ie, IC₅₀ concentration) was analyzed.

Western blot analysis

Breast cancer cells were used for Western blot analysis, as previously described.²⁵ Test compounds dissolved in dimethyl sulfoxide were added at indicated concentrations and cells were exposed to normoxic conditions $(21\% O_2)$ for 30 minutes. The plates were then incubated in hypoxic conditions $(1\% O_2)$ for 4 hours and harvested. Whole cell lysates were collected and 30 µg protein was subjected to Western blot analysis. The anti-HIF-1-alpha antibody (hydroxyl P402) was purchased from Abcam[®] (Cambridge, UK). The other primer antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

RNA extraction and quantitative real-time RT-PCR

For reverse transcription-polymerase chain reaction (PCR) analysis, total cellular RNA was isolated from the breast cancer cells with TRI reagent (Sigma-Aldrich Co., St Louis, MO, USA). The first-strand cDNA synthesis was carried out and PCR was performed on cDNA with $2\times$ PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and the corresponding primers. Quantitative real-time PCR (qRT-PCR) analyses were performed to determine the expression of *HIF-1* α , *VEGF*, *IGF-2*, and *GAPDH* (an internal control), as described by Niu et al.²⁶ qRT-PCR reactions were done in duplicate using KOD SYBR[®] qPCR Mix (Toyobo Co., Ltd). The PCR reaction was carried out on a thermocycler (Thermo Fisher Scientific; Model: 7900HT).

Enzyme-linked immunosorbent assay

For this assay, 5×10^4 MX-1 cells per well in 96-well plates were treated with test chemicals and then subjected to hypoxia for 24 hours. VEGF was quantitatively assessed from the supernatant medium by using the Human VEGF Quantikine enzyme-linked immunosorbent assay kit, following the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA).

shRNA transfections for VHL knockdown

Sequences for shRNA targeting VHL were synthesized and purchased from GenePharma (Shanghai, People's Republic of China). When the cells reached 90% confluence, they were transfected with shRNA plasmids pGPU6 following the manufacturer's instructions for Lipofectamine 2000 Reagent (Thermo Fisher Scientific). Then, the transfected cells were treated with LXY6090 for 24 hours and harvested for Western blotting. The DNA sequences of shVHL were as follows:

Forward: 5'-CACCGAAGAGTACGGCCCTGAA GAATTCAAGAGATTCTTCAGGGCCGTACTCTTC TTTTTTG-3'.

Reverse: 5'-GATCCAAAAAAGAAGAGTACGG CCCTGAAGAATCTCTTGAATTCTTCAGGGCCG TACTCTTC-3'.

Wound healing assay

T47D and MCF-7 cells were grown to full confluence in sixwell plates. After 24 hours, the cell monolayers were scratched using a sterile 200 μ L plastic pipette tip. Then, the cells were treated with LXY6090 and allowed to migrate to the scratched areas under hypoxic conditions. We captured five images from randomly chosen microscopic fields at indicated times. The area of the wound was measured by ImageJ software (National Institutes of Health, Bethesda, MD, USA), and an average value of the five areas was considered for further analyses.

In vivo studies

Cells from the human breast cancer cell line, MX-1, were cultured and harvested. Cells were resuspended in saline at 4×10⁶ cells/0.2 mL volume. The animal protocol was both followed and approved by the Experimental Animal Management and Welfare Committee at the Institute of Materia Medica, Peking Union Medical College. Athymic nude mice (BALB/c-nu/ nu females, 6–8 weeks old) were subcutaneously injected with 0.2 mL cells on the flanks. When the tumors grew to 100–200 mm³, 30 mice were randomly divided into five groups as follows: control group, Taxol group, 25 mg/kg LXY6090, 50 mg/kg LXY6090, and 100 mg/kg LXY6090. For control group, water was administered every day po. Taxol (paclitaxel injection) was purchased from Beijing Union Pharmaceutical Factory (Beijing, People's Republic of China). LXY6090 was dissolved in water and administered every day po for 14 days.

Immunohistochemistry

The HIF-1 α expression level of MX-1 tumor samples in the control and LXY6090 groups (50 mg/kg, 100 mg/kg) was detected using a 3,3'-diaminobenzidine (DAB)-based PV-9000-D kit (ZSGB-BIO, Zhong Shan Golden Bridge Biotechnology Co., Ltd, People's Republic of China), following the manufacturer's instructions. The primary antibody (Anti-HIF-1-alpha [H1alpha67]) was obtained from Abcam[®]. The quantitation of HIF-1 α was performed as described by Huang et al.²⁷

Statistical analysis

All statistical comparisons were carried out by GraphPad Prism[®] (Version 5.0, GraphPad Software, Inc., La Jolla, CA, USA) using the Student's unpaired *t*-test. For all tests, *P*-values < 0.05 were considered statistically significant.

Results

Identification of LXY6090 as a potent inhibitor of HIF-1

To explore novel small molecules targeting the HIF-1 pathway, the structure of MA (Figure 1A) was further modified and optimized based on the new derivatives - LXY6006 and LXY6099. The synthesized MA derivatives were screened using a cell-based dual luciferase reporter assay to enhance our knowledge about its structure-activity relationship. Among the 21 new compounds, we synthesized and initially screened the following: one compound (LXY6090) that had an IC_{50} value <10 nM, five compounds that had IC₅₀ values between 10 nM and 100 nM, eight compounds that had IC₅₀ values between 100 nM and 1,000 nM, and seven other compounds that had IC_{50} values >1,000 nM (Table 1). Compared with MA, LXY6090 has less chiral centers and is, therefore, easier to synthesize (Figure 1B). Furthermore, LXY6090 inhibited HIF-1 activity with an IC₅₀ value of 4.11±0.4 nM, which is much lower than that of MA (35.2±4.7 nM; Figure 1C).

LXY6090 downregulates HIF-1 α protein levels in multiple breast cancer cells

The ability of LXY6090 to regulate HIF-1 activity was tested by evaluating the protein levels of HIF-1 α in multiple breast cancer cells under hypoxia. The expression level of HIF-1 α proteins was low under normoxia, but it stabilized under



Figure I Chemical structures of MA derivatives and their effects on HIF-I activities in T47D cells.

Notes: The structure of MA and LXY6090, respectively (**A** and **B**). Dose-response studies were performed in T47D cells by a dual luciferase reporter assay to determine the effects of the compounds on HIF-I activities (**C**). MA was used as a positive control. After plasmid transfection, cells were exposed to various doses of test compounds for 30 min before hypoxia treatment (16 h, 1% O_2) and detected by a dual luciferase reporter assay. The HIF-I activities were defined as the ratio of renilla luciferase/firefly luciferase. Data are presented as mean \pm SD from one representative experiment performed in triplicate.

Abbreviations: h, hours; HIF-1, hypoxia-inducible factor-1; MA, manassantin A; min, minutes; SD, standard deviation.

hypoxia and became readily detectable by Western blotting in T47D, MCF-7, and MX-1 cells (Figure 2A), as described previously in other cancer cells.²⁸ The protein level of HIF-1 α was reduced in a dose-dependent manner after the cells' exposure to LXY6090 at the dose of 0.4–10 nM following 4 hours of hypoxia treatment (Figure 2A). In contrast to the robust effect on HIF-1 α protein level, LXY6090 had no effect on the HIF-1 β protein level as well as β -actin. To further test if transcriptional regulation was involved in HIF-1 α downregulation, the mRNA level of HIF-1 α was analyzed. As shown in Figure 2B, LXY6090 significantly decreased hypoxiainduced HIF-1 α mRNA in a dose-dependent manner. These results indicate that LXY6090 could inhibit HIF-1 α protein accumulation by decreasing the HIF-1 α mRNA.

LXY6090 inhibits HIF-1 α protein accumulation through proteasome degradation

Some HIF-1 inhibitors were identified to inhibit HIF-1 α not only by preventing its synthesis but also by promoting its degradation.^{26,29,30} Hence, we next examined the effect of LXY6090 on the degradation of HIF-1 α in MX-1 cells. As shown in Figure 3A, in the presence of MG132, a potent proteasome inhibitor, LXY6090, failed to downregulate hypoxia-induced HIF-1 α protein. This finding suggested

that LXY6090 could promote proteasome degradation of HIF-1 α under hypoxia. Because HIF-1 α hydroxylation and binding to VHL are necessary for its degradation,^{31,32} we further measured hydroxyl-HIF-1 α (pro564 and pro402) and VHL protein levels in these cells. As shown in Figure 3B, LXY6090 increased the level of hydroxylated HIF-1 α at pro564 and pro402 under both normoxic and hypoxic conditions, which was followed by an increased expression of VHL. To test if VHL is necessary for the downregulation of HIF-1a by LXY6090, VHL expression in MX-1 breast cancer cells was suppressed by a VHL shRNA knockdown analysis. As shown in Figure 3C, under hypoxic conditions, VHL expression was significantly knocked down by the shRNA approach (only 10% of the sh-control group). LXY6090 failed to suppress the accumulation of HIF-1 α in VHL knockdown cells, indicating that LXY6090 destabilized HIF-1 α by increasing VHL. Taken together, these results suggest that LXY6090 promotes HIF-1 a proteasome degradation in a VHL protein-dependent manner.

LXY6090 inhibits HIF-1 target genes and protein expression induced by hypoxia

To further confirm the inhibitory effect of LXY6090 on HIF-1, the HIF-1 target genes – VEGF and IGF-2 – were detected in MX-1 cells. The mRNA expression levels of

Table I Inhibition of HIF-1 by MA derivatives using a cell-based luciferase reporter assay

Range of IC ₅₀	Total	I–I0 nmol/L	10–100 nm/L	100–1,000 nmol/L	>1,000 nmol/L
Number	21	I	5	8	7
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Abbreviations: HIF-1, hypoxia-inducible factor-1; MA, manassantin A.



Figure 2 LXY6090 inhibits HIF-1 activity by preventing the accumulation of HIF-1 α protein and mRNA levels under hypoxia. Notes: After 4 h of hypoxia (1% O₂), in the presence of test compounds, HIF-1 α and HIF-1 β proteins levels of T47D, MCF-7, and MX-1 cells were determined by Western blotting. The images presented are from one representative experiment performed in triplicate. The quantification of HIF-1 α to actin and HIF-1 β to actin levels were normalized to the 1% O₂ group (C2 group) (**A**). HIF-1 α mRNA level was assessed in these three cell lines using qRT-PCR analysis (**B**). **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the 1% O₂ non-treated group (C2 group).

Abbreviations: h, hours; HIF-1, hypoxia-inducible factor-1; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

VEGF and *IGF-2* were downregulated after exposure to LXY6090 (Figure 4A). Consistently, the protein levels of both secreted and intracellular VEGF (VEGF₁₆₅ isoform) were reduced in a dose-dependent manner by LXY6090 under hypoxia (Figure 4B and C).

LXY6090 exhibits antitumor effect

in vitro

To investigate whether the inhibition of HIF-1 activity by LXY6090 would result in antitumor effects in breast cancer cells, the effects of LXY6090 on breast cancer cell growth, colony formation, and wound healing were assessed. The T47D, MCF-7, and MX-1 cell types were exposed to LXY6090 at various concentrations ranging from 10^{-4} M to 10^{-9} M for 96 hours, and the cell viability was determined by the MTT assay. As expected, LXY6090 effectively inhibited the growth of these breast cancer cells, and the effect was much stronger than MA (Figure 5A; Table 2). Moreover, LXY6090 appeared

to inhibit the colony formation of T47D and MCF-7 cells more effectively than MA (Figure 5B). The wound healing assay was performed to study directional cell migration in vitro. As shown in Figure 5C, LXY6090 could prevent wound healing in T47D and MCF-7 cells at 24 h/48 h at a concentration of 2 nM under hypoxic conditions. These three assays imply that LXY6090 has a considerable in vitro antitumor effect.

LXY6090 inhibits the growth of MX-I xenograft tumor in nude mice

To determine whether LXY6090 was also effective against tumors in vivo, the antitumor effect of LXY6090 was studied against the MX-1 subcutaneous xenograft tumors in nude mice. Water was used as a control for LXY6090. Taxol, a well-known chemotherapeutic agent, was intraperitoneally injected into mice as a positive control for comparing and contrasting the efficacy of LXY6090. Oral administration of LXY6090 (25 mg/kg/d, 50 mg/kg/d, and 100 mg/kg/d)



Figure 3 LXY6090 inhibits HIF-1 α protein accumulation by promoting its degradation.

Notes: MX-1 cells were treated with LXY6090 in the presence of 1 μ M MG132 for 30 min and then cultured under hypoxic conditions for an additional 4 h. Whole cell extracts were prepared for Western blotting. The representative photographs and the quantification of HIF-1 α /actin and VHL/actin levels normalized to the 21% O₂ group are shown (**A**). MX-1 cells were treated with/without LXY6090 at normoxic and hypoxic conditions; the hydroxyl-HIF-1 α (Pro402 and Pro564), HIF-1 α , and VHL proteins were determined by Western blotting. The representative photographs and the quantification of OH-HIF-1 α /HIF-1 α and VHL/actin levels normalized to the 21% O₂ group at each condition are shown. ***P<0.001 compared with the 21% O₂ non-treated group. #P<0.05, ##P<0.01, ###P<0.001 compared with the 1% O₂ non-treated group (**B**). VHL protein was knocked down by the shRNA method. Then, under hypoxic conditions, the effect of LXY6090 on VHL and HIF-1 α was assessed by Western blotting. The representative and VHL/actin levels normalized to the 11% O₂ non-treated group (**B**). VHL protein was knocked down by the shRNA method. Then, under hypoxic conditions, the effect of LXY6090 on VHL and HIF-1 α was assessed by Western blotting. The representative experiment performed in triplicate. *P<0.05, **P<0.01 compared with the shRNA-control group (**C**). **Abbreviations:** h, hours; HIF-1, hypoxia-inducible factor-1; min, minutes; VHL, von Hippel–Lindau.

significantly reduced the tumor volume in a dose-dependent manner, suggesting that LXY6090 was effective at inhibiting the growth of breast cancer MX-1 xenograft tumors (Figure 6A). After tumors were harvested and weighed on day 14, the LXY6090-treated xenograft tumors were significantly lighter than those in the control group. In brief, LXY6090 at doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg resulted in approximately 28%, 39%, and 60% inhibitions, respectively (Figure 6B). The antitumor effect of LXY6090 at the dose of 50 mg/kg was similar to that of Taxol (Figure 6B).



Figure 4 LXY6090 inhibits hypoxia-induced VEGF/IGF-2 gene and protein expression.

Notes: The MX-I cells were exposed to test compounds for 30 min prior to hypoxic exposure (20 h, $1\% O_2$). VEGF and IGF-2 mRNA levels were estimated in MX-I cells by using qRT-PCR analysis. Data shown are relative expression levels (mean \pm SD) of VEGF normalized to GAPDH control, determined from one representative experiment performed in triplicate (**A**). Secreted VEGF proteins were determined by ELISA and normalized to the C1 group (21% O_2 group) (**B**). The cellular VEGF protein levels were tested by Western blotting (**C**). *P<0.05, **P<0.01, ***P<0.001 compared with the C2 group (1% O_2 non-treated group).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; h, hours; MA, manassantin A; min, minutes; VEGF, vascular endothelial growth factor; SD, standard deviation; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

The body weights of mice did not show any obvious difference among the different groups, suggesting that LXY6090 had minimal effect on body weights (Figure 6C). As Taxol is known for its inhibitory effect on bone marrow-originated blood cell production, we further examined the peripheral white blood cell (WBC) counts in these mice. As shown in Figure 6D, the WBC count in the Taxol group was significantly lower than that in the control group. Interestingly, mice that underwent LXY6090 treatment had similar WBC counts as mice in the control group (Figure 6D). These data indicated that LXY6090 at the tested doses was safe to mice during the 14 days of experiment.

LXY6090 inhibits HIF-1 α expression in vivo

To further investigate whether the antitumor effect of LXY6090 was due to the decreased expression level of HIF-1 α ,

the harvested tumors were lysed and the homogenates were subjected to Western blot analysis to examine the HIF-1 α level in the MX-1 tumor tissues. Indeed, LXY6090 decreased the HIF-1 α level in a dose-dependent manner (Figure 7A). Furthermore, immunohistochemistry results revealed that tumor tissues treated with LXY6090 showed significantly lower expression levels of HIF-1 α than that of the corresponding control tissue (Figure 7B).

Discussion

The dineolignan MA, isolated from the aquatic plant *S. cernuus* L. (family: Saururaceae), is reportedly a potent inhibitor of HIF-1.^{17,18} Like several other HIF-1 α inhibitors,³³ MA can enhance cancer cells' sensitivity to chemotherapy and radiotherapy through HIF-1 α inhibition. Therefore, the combination of MA and other cytotoxic drugs and/or radiation could have great potential in



Figure 5 Inhibitory effects of LXY6090 on breast cancer cells.

Notes: The efficacies of LXY6090 on the breast cancer cell lines were determined by MTT assay (**A**) and colony formation (**B**) under normoxic conditions. Wound areas were measured at 0 h, 24 h, and 48 h in the presence of different concentrations of LXY6090. (**C**) The concentration of MA is 100 nM in both (**B**) and (**C**). *P<0.05, **P<0.01, and ***P<0.001 compared with the control group at 24 h and 48 h.

Abbreviations: h, hours; MA, manassantin A.

enhancing cancer therapy. However, complexities in the total chemical synthesis of MA because of its eight chiral centers limit its application in in vivo antitumor research. In this study, we developed a novel MA derivative, LXY6090, with the aim of easy synthesis and more potent anti-breast cancer targeting HIF-1-mediated pathways. Specifically, the tetrahydrofuran moiety in MA was replaced by a cyclopentane core to reduce the number of chiral centers and preserve the main structure. This modification enabled the total chemical synthesis and purification of LXY6090 in our laboratory.

Breast cancer is the most common malignancy in women, and HIF-1 α protein is overexpressed in many primary breast cancers.³⁴ HIF-1 α expression levels can increase in human breast cancer, thereby altering the cancer's molecular

 Table 2 Effect of LXY6090 on breast cancer cell growth

Compound	Growth inhibit		
	T47D	MCF-7	MX-I
MA	1,054.4±105.2	1,262.7±156.9	879.8±13.8
LXY6090	245.7±15.2	352.7±14.2	108.2±2.1

Note: Data are presented as mean \pm standard deviation **Abbreviation**: MA, manassantin A.



Figure 6 LXY6090 inhibits MX-I xenograft tumor growth in vivo.

Notes: As a control, Taxol was administered to nude mice bearing MX-1 xenograft tumors by ip at a dose of 20 mg/kg, every 3 d, four times in all. Additionally, LXY6090 was orally administered at doses of 25 mg/kg/d, 50 mg/kg/d, and 100 mg/kg/d (one dose each day). Tumor volumes were measured at the indicated time (**A**) and tumors were measured after the mice were sacrificed (**B**). Animals were also monitored for changes in body weight (**C**) and peripheral blood WBC (**D**) as surrogate markers for toxicity. *P<0.05 vs control, **P<0.01, **P<0.001 vs control.

Abbreviations: d, days; ip, intraperitoneally; WBC, white blood cell.

pathogenesis. As previously reported, the MA derivative, LXY6006, had an inhibitory effect on breast cancer through HIF-1;²³ therefore, we chose three breast cancer cell lines, all expressing HIF-1 α protein as our therapy target for MA derivatives. Here, we identified another novel MA derivative, LXY6090, as a potent HIF-1 inhibitor in these breast cancer cells. LXY6090 not only inhibited the activity of HIF-1 but also downregulated hypoxia-induced protein expression and the gene level of HIF-1 α (Figure 2).

The reduction in HIF-1 α protein may occur via the decrease of HIF-1 α synthesis or the increase of HIF-1 α degradation. Our study indicated that LXY6090 decreased HIF-1 α protein accumulation by inhibiting the protein synthesis (decreased mRNA level). Next, we focused on

the HIF-1 α degradation pathway. It was interesting to note that LXY6090 regulates HIF-1 α protein by activating the proteasome degradation, as shown by the MG132 study (Figure 3A). It was further confirmed that hydroxyl-HIF-1 α was increased upon LXY6090 treatment through VHL protein (Figure 3B and C). These results suggested that LXY6090 promotes proteasome degradation of HIF-1 α , which may depend on VHL protein.

As reported previously, HIF-1 inhibitors have been developed as antitumor agents.³³ LXY6090 was also shown to effectively decrease breast cancer growth both in vitro and in vivo. LXY6090 could inhibit the growth of T47D, MCF-7, and MX-1 cells under normoxic conditions (Figure 5). We could not carry out the same MTT assay under hypoxic conditions,



Figure 7 Effects of LXY6090 on HIF-1 a expression in vivo.

Notes: The expression levels of HIF-1 α in MX-1 tumor tissues are shown by a representative Western blot, and quantitative data (mean \pm SD) are calculated based on at least three independent experiments (**A**). Histological analysis (HIF-1 α) of tumors is shown by representative photographs (**B**). Magnification: ×20. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control.

Abbreviations: HIF-1, hypoxia-inducible factor-1; SD, standard deviation.

because the cells did not grow under hypoxia. Among the three breast cancer cells, MX-1 is the only estrogen-negative cell type and is, hence, relatively more difficult to cure than the other cell types. However, LXY6090 was most effective on MX-1 cells, which indicates that LXY6090 can be applied to treat estrogen-negative breast cancer. We were further encouraged to explore the antitumor effect of LXY6090 on rapid-growing MX-1 xenograft models in vivo (Figure 6). Compared with Taxol, LXY6090 had less toxicity on WBCs, but could significantly decrease MX-1 growth in vivo, suggesting that LXY6090 alone could be used for breast cancers or in combination with other chemotherapeutic drugs.

As MA is reportedly an HIF-1 inhibitor, there are numerous efforts to simplify its structure.^{16,20,35} To the best of our knowledge, our study is the first to replace the central tetrahydrofuran moiety of MA with a cyclopentane ring in both LXY6006 and LXY6099.^{22,23} Compared with the published LXY6006 compound, LXY6090 and LXY6099 are much easier to synthesize because of their amide bond structure. Moreover, LXY6090 has a much lower molecular mass than LY6099, which is more suitable for drug development. Although there are several MA analogs, the HIF-1 inhibition mechanisms of these compounds are still undiscovered. Herein, we first indicated that LXY6090 suppressed HIF-1 activity by promoting HIF-1 α degradation.

Further studies are required to reveal the detailed mechanism of how LXY6090 affects VHL or other related proteins such as the prolyl-4-hydroxylases. Therefore, in our next study, we will likely focus on the HIF-1 α degradation pathway, in addition to exploring LXY6090's metabolites and pharmacokinetics.

Conclusion

In this study, we identified a synthetic MA derivative, LXY6090, as a novel HIF-1 inhibitor that has the potential to be further developed into a novel antitumor agent, especially for

human breast cancers. Further detailed studies are underway to clarify the mechanism of LXY6090 in breast cancers.

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Disclosure

The authors report no conflicts of interest in this work.

References

- Brahimi-Horn MC, Chiche J, Pouyssegur J. Hypoxia and cancer. J Mol Med (Berl). 2007;85(12):1301–1307.
- Chiche J, Ricci JE, Pouysségur J. Tumor hypoxia and metabolism towards novel anticancer approaches. *Ann Endocrinol (Paris)*. 2013; 74(2):111–114.
- Brahimi-Horn MC, Pouysségur J. The hypoxia-inducible factor and tumor progression along the angiogenic pathway. *Int Rev Cytol.* 2005; 242:157–213.
- 4. Kaelin WG Jr. Cancer and altered metabolism: potential importance of hypoxia-inducible factor and 2-oxoglutarate-dependent dioxygenases. *Cold Spring Harb Symp Quant Biol.* 2011;76:335–345.
- Mimeault M, Batra SK. Molecular biomarkers of cancer stem/progenitor cells associated with progression, metastases, and treatment resistance of aggressive cancers. *Cancer Epidemiol Biomarkers Prev.* 2014;23(2): 234–254.
- Wang GL, Semenza GL. Purification and characterization of hypoxiainducible factor 1. *J Biol Chem*. 1995;270(3):1230–1237.
- Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A*. 1995;92(12):5510–5514.
- Ivan M, Kondo K, Yang H, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*. 2001;292(5516):464–468.
- Maxwell PH, Wiesener MS, Chang GW, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*. 1999;399(6733):271–275.
- Bruick RK. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes Dev.* 2003; 17(21):2614–2623.
- Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redoxinduced changes. *J Biol Chem.* 1997;272(36):22642–22647.
- Semenza GL. HIF-1 and human disease: one highly involved factor. Genes Dev. 2000;14(16):1983–1991.
- Shannon AM, Bouchier-Hayes DJ, Condron CM, Toomey D. Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. *Cancer Treat Rev.* 2003;29(4):297–307.
- Onnis B, Rapisarda A, Melillo G. Development of HIF-1 inhibitors for cancer therapy. J Cell Mol Med. 2009;13(9A):2780–2786.
- Ward C, Langdon SP, Mullen P, et al. New strategies for targeting the hypoxic tumour microenvironment in breast cancer. *Cancer Treat Rev.* 2013;39(2):171–179.

- Kwon DY, Lee HE, Weitzel DH, et al. Synthesis and biological evaluation of manassantin analogues for hypoxia-inducible factor 1alpha inhibition. *J Med Chem.* 2015;58(19):7659–7671.
- Hodges TW, Hossain CF, Kim YP, Zhou YD, Nagle DG. Moleculartargeted antitumor agents: the *Saururus cernuus* dineolignans manassantin B and 4-O-demethylmanassantin B are potent inhibitors of hypoxia-activated HIF-1. *J Nat Prod.* 2004;67(5):767–771.
- Hossain CF, Kim YP, Baerson SR, et al. Saururus cernuus lignans potent small molecule inhibitors of hypoxia-inducible factor-1. Biochem Biophys Res Commun. 2005;333(3):1026–1033.
- Kasper AC, Moon EJ, Hu X, et al. Analysis of HIF-1 inhibition by manassantin A and analogues with modified tetrahydrofuran configurations. *Bioorg Med Chem Lett.* 2009;19(14):3783–3786.
- Minegishi H, Matsukawa T, Nakamura H. Synthesis and biological evaluation of diaryl-substituted carboranes as inhibitors of hypoxia inducible factor (HIF)-1 transcriptional activity. *ChemMedChem*. 2013; 8(2):265–271.
- Rao KV, Puri VN, Diwan PK, Alvarez FM. Preliminary evaluation of manassantin A, a potential neuroleptic agent from *Saururus cernuus*. *Pharmacol Res Commun*. 1987;19(9):629–638.
- 22. Lai FF, Liu XY, Niu F, Lang LW, Xie P, Chen XG. [A novel HIF-1 inhibitor manassantin A derivative LXY6099 inhibits tumor growth]. *Yao Xue Xue Bao*. 2014;49(5):622–626. Chinese.
- 23. Lang L, Liu X, Li Y, et al. A synthetic manassantin a derivative inhibits hypoxia-inducible factor 1 and tumor growth. *PLoS One*. 2014;9(6):e99584.
- Lang L, Tang K, Li Y, Liu X, Wang C, Chen X. [Identification of two small molecule inhibitors of hypoxia-inducible factor 1 with different cell-based screening model]. *Zhongguo Zhong Yao Za Zhi*. 2012; 37(14):2151–2155. Chinese.
- Lai F, Madan N, Ye Q, et al. Identification of a mutant alphal Na/K-ATPase that pumps but is defective in signal transduction. *J Biol Chem.* 2013;288(19):13295–13304.
- Niu F, Li Y, Lai FF, et al. LB-1 exerts antitumor activity in pancreatic cancer by inhibiting HIF-1alpha and Stat3 signaling. *J Cell Physiol.* 2015;230(9):2212–2223.
- Huang D, Ding Y, Zhou M, et al. Interleukin-8 mediates resistance to antiangiogenic agent sunitinib in renal cell carcinoma. *Cancer Res.* 2010; 70(3):1063–1071.
- Sun HL, Liu YN, Huang YT, et al. YC-1 inhibits HIF-1 expression in prostate cancer cells: contribution of Akt/NF-kappaB signaling to HIF-1alpha accumulation during hypoxia. *Oncogene*. 2007;26(27): 3941–3951.
- Cao Z, Fang J, Xia C, Shi X, Jiang BH. trans-3,4,5'-Trihydroxystibene inhibits hypoxia-inducible factor 1 alpha and vascular endothelial growth factor expression in human ovarian cancer cells. *Clin Cancer Res.* 2004; 10(15):5253–5263.
- Zhang Q, Tang X, Lu Q, Zhang Z, Rao J, Le AD. Green tea extract and (-)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1alpha protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. *Mol Cancer Ther*. 2006;5(5): 1227–1238.
- Kondo K, Kaelin WG Jr. The von Hippel-Lindau tumor suppressor gene. Exp Cell Res. 2001;264(1):117–125.
- Mole DR, Maxwell PH, Pugh CW, Ratcliffe PJ. Regulation of HIF by the von Hippel-Lindau tumour suppressor: implications for cellular oxygen sensing. *IUBMB Life*. 2001;52(1–2):43–47.
- Masoud GN, Li W. HIF-1alpha pathway: role, regulation and intervention for cancer therapy. *Acta Pharm Sin B*. 2015;5(5):378–389.
- 34. Gilkes DM, Semenza GL. Role of hypoxia-inducible factors in breast cancer metastasis. *Future Oncol.* 2013;9(11):1623–1636.
- 35. Li J, Zhao K, Wang F, Cai J, Li Z, Zou L. X609, a novel manassantin A derivative, exhibits antitumor activity in MG-63 human osteosarcoma cells in vitro and in vivo. *Mol Med Rep.* 2015;12(2):3115–3120.

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